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Research Article

MITOCHONDRIAL COI GENE-BASED MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS IN THIRTEEN DRAGONFLIES (ODONATA: LIBELLULIDAE) OF BANGLADESH

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ABSTRACT. Dragonflies serve as essential predators in Integrated Pest Management (IPM). Nevertheless, polymorphism, sexual dimorphism, and body color changes make dragonfly species identification difficult. The application of the mitochondrial cytochrome c oxidase I (COI) gene to identify dragonflies is effective. In the present study, COI gene sequences of thirteen species from the family Libellulidae were generated. The COI gene sequences of twelve dragonfly species were 98% to 99% similar to the gene sequences of their respective species, according to BLAST analysis. Only one species, *Brachydiplax farinosa*, did not match significantly to any sequences in GenBank. Then, all thirteen sequences were submitted to GenBank, where only *B. farinosa*'s sequence was submitted for the first time. Using MEGA10 and BioEdit, a 569bp COI gene fragment with 369 conserved sites, 200 variable sites, and 167 parsimony-informative sites was identified. The average base composition of the *COI* sequences was 35.43% T, 17.65 % C, 29.83% A, and 17.07% G. While there was a significant AT bias (65.26%) among dragonfly species. Pairwise distance analysis and phylogenetic tree was used to explore genetic diversity and evolutionary relationships among dragonfly species. The thirteen dragonfly species had 0.119-0.231% interspecific genetic divergence. A phylogenetic tree using the Neighbor-Joining (NJ) algorithm revealed two major clades, A and B, and demonstrated that all dragonfly species shared a common ancestor. These two clades encompass all twelve species, except *Tholymis tillarga*. Subsequently, a TCS haplotype network unveiled the genetic relatedness of these thirteen dragonflies, with *T. tillarga* exhibiting the highest number of mutations (49) in the analysis. *T. tillarga* has experienced significant genetic adaptation over time and could be an excellent model organism to study numerous biological processes. In addition, this work also supports to build a complete DNA barcode database in GenBank and uses DNA barcoding to identify thirteen dragonflies from Bangladesh.

Keywords: *Dragonfly, COI gene, Identification, Phylogeny, Bangladesh.*

INTRODUCTION

The insect order Odonata has two suborders, the Anisoptera and the Zygoptera, which are composed of dragonflies and damselflies, respectively. The Libellulidae is one of the largest family in the suborder Anisoptera. Libellulidae has 1035 species in 144 genera all over the world, and Bangladesh has 49 species in 28 genera [1-7]. The wings of most libellulids are brightly coloured and intricately patterned, and their hind wings typically feature an anal loop. Some species are even able to thrive in brackish water and others in water with low oxygen levels [8]. They serve as predators in aquatic and terrestrial environments, where they prey on small insects such as moths, chironomid midges, and beetles that are detrimental to various crops. Their nymphs feed on the larvae of numerous species of mosquitoes, hence the name "mosquito hawks" [9]. Dragonflies are effective bioindicators due to their ability to detect and rapidly respond to environmental changes [10]. Thus, they necessitate particular habitat, and their presence reveals environmental conditions [11].

Nonetheless, the characterization and identification of these dragonflies' taxa are essential to biomonitoring programmes in order to comprehend ecosystem health and preserve dragonflies as potential bioindicators [12, 13]. However, the most difficult aspect of these approaches is the correct identification of the dragonflies. This is because dragonflies have polymorphic morphologies, which include differences in body size, wing shape, and coloration, challenging to recognize individual species. Furthermore, the presence of distinct colour differences between males and females in various anatomical regions serves as exemplification of sexual dimorphism. One category of dragonflies that display variations in body coloration are the newly emerged adult dragonflies, sometimes referred to as "tenerals." These individuals often showcase hues that are somewhat subdued and less vibrant in comparison to their fully developed counterparts [14]. Consequently, conventional identification techniques of dragonflies based on morphology can be difficult and time-consuming, leading to misidentification. Moreover, the colour patterns of these insects from different environments or regions may vary. In this context, DNA barcoding using COI gene is a more accurate and effective method for species identification that can reduce misidentification, improve biodiversity monitoring, and facilitate evolutionary research [15-18]. In addition, the application of the COI gene aids in the identification of cryptic species among dragonflies and allows for the differentiation of genetically diverse populations [19-22].

Besides accurate identification, their unusual mating behavior and intricate reproductive structure, dragonflies have been used as model organisms in numerous ecological and evolutionary studies [23-28]. The construction of a phylogenetic tree based on COI genes allowed researchers to better understand the origins and evolutionary relationships of dragonfly species [29-33]. Furthermore, many Odonates families, including the Libellulidae, could not resolve their phylogenetic positions [34]. Therefore, the present study was carried out to generate the molecular marker, COI gene for species identification for biodiversity monitoring and to study the phylogenetic relationship among the species of Libellulids.

MATERIALS AND METHODS

Sample collection

A total of thirteen dragonfly specimens were collected from diverse locations in Bangladesh (Table 1). Following their collection in the field using an insect net, the samples were dehydrated and enclosed in a small envelope to ensure their preservation. To identify dragonflies, morphological keys developed by Fraser [35, 36, 37], Subramanian and Babu [5], Mitra [38] and Lahiri [39] were used. Brower [40] provided the guidelines for the creation of voucher samples.

DNA extraction, amplification and sequencing

Following the protocol specified in the Wizard Genomic@ DNA Purification Kit, genomic DNA was extracted from the legs of thirteen different dragonfly species. Primers LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) were used for PCR amplification of COI gene region. Q2 Green PCR Master Mix was used in an Applied Biosystems Veriti thermal cycler to perform polymerase chain reaction. The PCR reaction mixture comprised the following components: 10 µl of 2x master mix, 1 µl each of forward and reverse primers (10 pM), template DNA (amount dependent on the quantity of extracted DNA), and 7 µl of water that is free of nuclease, for a total volume of 20 µl. The process began with a 5-minute stage of initial denaturation at a temperature of 95°C. This was followed by 35 cycles consisting of a 30-second denaturation at 95°C, a 30-second primer annealing at 49°C, and a 45-second primer extension at 72°C. The cycle ended with a concluding extension step at a temperature of 72°C for a duration of 5 minutes. The evaluation of the amplification's effectiveness was achieved by utilizing 1% agarose gel electrophoresis under UV light conditions, using a Bio Analyzer. The amplified product underwent sequencing analysis on an ABI 3500 sequencer.

Genetic distance and phylogenetic analysis

Chromas 2.6.2 was utilized to modify the sequences of these thirteen species of dragonflies. A ClustalW multiple alignment program integrated into BioEdit version 7.0 was utilized to align the assembled sequences [41]. In this study, the Kimura 2-Parameter (K2P) model was employed in conjunction with the MEGA10 software to calculate and summarize nucleotide compositions, as well as to estimate pairwise distances [42, 43]. By employing 1000 bootstrap replications, a phylogenetic tree was constructed using the Neighbor-joining (NJ) method in MEGA10 [43, 44]. *Aedes aegypti* (OP942188) was utilized as an out group in the phylogenetic analysis. Furthermore, the inclusion of additional genes from the GenBank database was undertaken for the purpose of enhancing the study, as depicted in Figure 1.

RESULTS AND DISCUSSION

In the current investigation, mitochondrial COI gene sequences (mtCOI) spanning around 636 bp were generated from thirteen different species of dragonflies that belong to the family Libellulidae. The BLAST analysis revealed that the COI gene sequences of these dragonflies were 98% to 99% similar to the gene sequences of their respective species. The only species that didn't have a strong similarity was *B. farinosa*, whose

sequences didn't match any in the GenBank database. After that, all thirteen sequences were submitted to GenBank, and received accession numbers (Table 1). The sequence of *B. farinosa* was the only one that was submitted for the first time in the GenBank database.

Table 1. *Sequenced species and GenBank accession numbers for COI genes*

Sl	Scientific name	Geo location	Voucher no.	Accession no.
01	*Acisoma panorpoides*	23.87442 N 90.26814 E	DRBV 0037	MN689142
02	*Brachydiplax chalybea*	23.87442 N 90.26925 E	DRBV 0032	MN689144
03	*Brachydiplax farinosa*	23.87742 N 90.26981 E	DRBV 0033	MN689143
04	*Brachydiplax sobrina*	23.87247 N 90.26667 E	DRBV 019	MH019981
05	*Bradinopyga geminata*	23.87414 N 90.26731 E	DRBV 044	MH019980
06	*Neurothemis fulvia*	23.87414 N 90.26731 E	DRBV 015	MH019979
07	*Neurothemis tullia*	23.8758 N 90.2683 E	DRBV 039	MH019982
08	*Orthetrum glaucum*	23.87614 N 90.26475 E	DRV 028	OR287446
09	*Orthetrum sabina*	23.87274 N 90.26757 E	DRBV 029	MF784360
10	*Rhodothemis rufa*	23.8758 N 90.2683 E	DRBV 022	MH019983
11	*Tramea basilaris*	23.87500 N 90.26472 E	DRBV 0046	MK779169
12	*Tholymis tillarga*	23.87500 N 90.26472 E	DRBV 012	MH019978
13	*Trithemis pallidinervis*	22.50272 N 92.15281 E	DRV 020	OR287444

Nucleotide analysis of COI gene

BioEdit and MEGA10 were used to evaluate the COI gene sequences, and the results revealed a 569 bp COI gene fragment with 369 conserved sites, 200 variable sites, and 167 parsimony-informative sites (Table 2). The majority of changes were seen at the second codon position. In Table 2, it is seen that out of the informative sites, 54 were ranked in the first position, 65 were ranked in the second position, and 48 were ranked in the third position. Generally for other protein-coding genes, the majority of variants were observed at the third codon position [45]. The current investigation demonstrated differences occurring at the second codon positions. In phylogenetics, the sequence analysis of these three different sites is crucial for understanding the evolutionary relationships among organisms [46]. Among these three sites, parsimony-informative sites are a subset of variable sites that exhibit different nucleotides or amino acids in such a way that they help to resolve the branching pattern in a phylogenetic tree with minimal evolutionary changes [47]. An analysis of the COI sequences' average base compositions revealed the following values: 35.43% thymine (T), 17.65% cytosine (C), 29.83% adenine (A), and 17.07% guanine (G) (Table 2). A substantial bias was observed for the adenine-thymine (A+T) base pair, which made up 65.26% of all base pairs. The adenine-thymine (A+T) content of the first, second, and third codon positions of the cytochrome c oxidase subunit I (COI) fragment was found to be 58.38%, 86.03%, and 51.32%, respectively (Table 2). It is important to note that the degree of A+T bias can vary substantially between organisms and genomic regions. In order to better understand the

biology and evolution of organisms, as well as to gain insight into the functional significance of particular genomic regions, researchers frequently examine these biases. In this context, Simon et al. [48] discovered that insect mitochondrion genomes are typically highly A+T biased, as in the present study. In addition, previous studies on the mitochondrial genomes of *Drosophila yakuba* (78.6%) and honey bees (84%) are consistent with the current investigation's findings (65.26%) (Table 2) [49, 50].

Table 2. *Basic statistics for COI gene sequences in thirteen dragonfly species*

Position	No. of Sites	No. Conserved Sites	No. Variable Sites	No. Parsimony Informative	Statistical base frequencies (%)				AT %
					T	C	A	G	
All Positions	569	369	200	167	35.43	17.65	29.83	17.07	65.26
First Position	190	128	62	54	44.17	26.84	14.21	14.77	58.38
Second Position	190	116	74	65	39.27	8.62	46.76	05.34	86.03
Third Position	189	125	64	48	22.79	17.50	28.53	31.17	51.32

Analysis of genetic distance

With the help of the MEGA10 programme, the pairwise distance was determined. Thirteen sequences of Libellulidae dragonflies had genetic distances ranging from 0.119 to 0.231% (Table 3). The lowest genetic distance (0.119%) was found between *Rhodothemis rufa* and *Acisoma panorpoides.* While the highest genetic distance (0.231%) was found between *Tramea basilaris and Neurothemis fulvia* (Table 3). Most animals showed intraspecific divergences are rarely greater than 2%, and most are less than 1% [51, 52]. In the present study, low interspecies divergence was observed, (0.119% to 0.231%), which indicates interspecies hybridization [45]. Interspecies hybridization is a well-known phenomenon in many insects, including dragonflies [53, 54]. The factor like genetic similarity contributes to hybridization in dragonflies and influences the genetic diversity of dragonfly populations [55]. Moreover, environmental factors like habitat heterogeneity and climate change can influence dragonfly genetic divergence [56, 57]. Understanding these factors is crucial for effective conservation and management of dragonfly populations as well.

Table. 3. *Percentage pairwise distances among thirteen dragonfly species*

	1	2	3	4	5	6	7	8	9	10	11	12
01. ***Acisoma panorpoides***												
02. ***Brachydiplax chalybea***	0.171											
03. ***Brachydiplax farinosa***	0.182	0.179										
04. ***Bradinopyga geminata***	0.184	0.185	0.186									
05. ***Brachydiplax sobrina***	0.183	0.184	0.142	0.160								
06. ***Neurothemis fulvia***	0.197	0.215	0.192	0.198	0.189							
07. ***Neurothemis tullia***	0.185	0.191	0.210	0.189	0.192	0.154						
08. ***Orthetrum glaucum***	0.151	0.177	0.147	0.155	0.172	0.192	0.191					
09. ***Orthetrum sabina***	0.174	0.191	0.170	0.166	0.163	0.195	0.192	0.123				
10. ***Rhodothemis rufa***	0.119	0.182	0.206	0.154	0.193	0.215	0.200	0.176	0.189			
11. ***Tramea basilaris***	0.146	0.200	0.178	0.156	0.185	0.231	0.205	0.176	0.195	0.162		
12. ***Tholymis tillarga***	0.176	0.195	0.182	0.179	0.193	0.200	0.204	0.168	0.185	0.195	0.202	
13. ***Trithemis pallidinervis***	0.154	0.211	0.159	0.146	0.158	0.203	0.185	0.137	0.140	0.167	0.163	0.164

Phylogenetic analysis

The neighbor-joining (NJ) phylogenetic tree constructed from the COI gene sequences of the thirteen dragonflies revealed that all thirteen species were monophyletic entities with a common ancestor (Fig. 1). A and B are the two principal clades disclosed by the phylogenetic tree. In the present study, the bootstrap value among all thirteen species with their corresponding species from different geographic origins was 99, indicating their c*lose genetic relationship (Fig. 1).* Within Clade A, there were four species, which were composed of *A. panorpoides, R. rufa, T. basilaris and B. geminata.* Among these species, 83 bootstrap value revealed a strong relationship between *A. panorpoides* and *R. rufa*. On the other hand, *T. basilaris* demonstrated a moderate bootstrap value of 69 with *A. panorpoides* and *R. rufa.* While *A. panorpoides, R. rufa,* and *T. basilaris* provided weak support for *B. geminata.*

Then again, Clade B consisted of eight species, which included *N. fulvia, N. tullia, B. chalybea, B. sobrina, B. farinosa, O. sabina, O. glaucum,* and *T. pallidinervis.* Among them, *N. fulvia* and *N. tullia* demonstrated a strong relationship, having a bootstrap value of 98. Conversely, *B. chalybea* exhibited a bootstrap value of 37, indicating a lower level of support with *N. fulvia* and *N. tullia.* Whereas *B. sobrina* and *B. farinosa* demonstrated a robust correlation with a bootstrap value of 71. The relationship between *O. sabina* and *O. glaucum* is moderate, with a bootstrap value of 68. On the other hand, a weak support exists among *T. pallidinervis, O. sabina,* and *O. glaucum*. In addition, *T. tillarga* is located far apart from both clades, with a bootstrap value of 34, suggesting a different genetic composition from the species of 2 other clades. There has been much debate over the precise phylogenetic location of the Odonata within the evolutionary arrangement of its member families, despite a rich fossil record and a large number of relatively recent phylogenetic analyses [58-64].

Dragonflies belonging to the family Libellulidae are involved in the assessment of phylogenetics and evolutionary history across various geographical regions [65]. According to a study by Nisar et al. [65], a phylogenetic analysis of ten species of dragonflies using the mitochondrial gene (12S rRNA) identified two groups whose pattern of results supported our findings. Laltanpuii et al. [66] conducted another analysis on eighteen genera under Libellulidae that include *Trithemis, Neurothemis, Tramea* as

well as *Orthetrum* and found that they formed monophyletic groups. Our findings align with this conclusion, except *T. tillarga, which* was found to be distantly located than two clades on the tree (Fig. 1). Although Bangladesh is home to forty-nine species of Libellulidae, only thirteen species of COI gene were generated for phylogenetic analysis in the present study [1-4]. A thorough phylogenetic analysis is required in order to acquire a complete understanding of the evolutionary processes and interrelationships among various species of Libellulidae. This will necessitate future research involving a greater variety of loci and species.

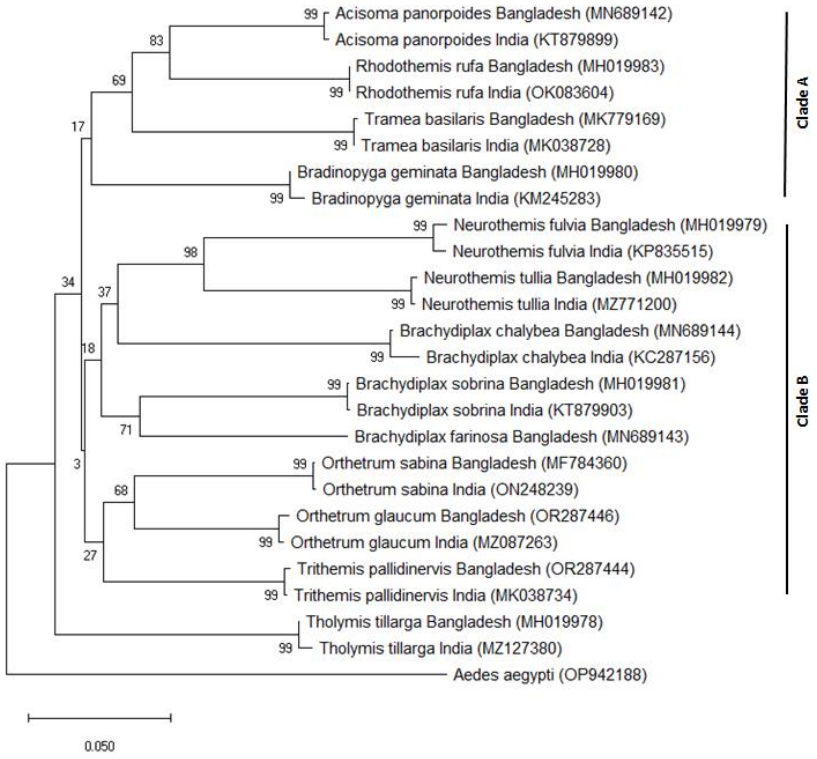

Fig. 1*. The Neighbor-Joining tree of the thirteen dragonflies is based on partial sequences of the mitochondrial COI gene. A. aegypti was used as the outgroup.*

Haplotype network

The TCS haplotype network of the mitochondrial COI gene in thirteen dragonflies revealed the mutational links between them (Fig. 2). The highest number of mutated sites (49 steps) among all dragonflies showed in *T. tillarga* indicating that this taxon's genetic makeup is different. *T. tillarga* and *B. geminata* separated from its immediate common ancestor by 49 and 40 mutational steps, respectively. These two species were separated again from *T. pallidinervis* by 33 mutational sites. Besides *T. tillarga*, the highest number of mutation occurs in *N. fulvia*, *B. chalybea* and *T. basilaris*, whose number is 40, 47 and 42 respectively. While *B. sobrina* and *B. farinosa* diverged from their closest common ancestors by 42 and 31 mutation steps, respectively (Fig. 2).

Nonetheless, in the present study, the highest number of mutations in *T. tillarga*, along with being distantly positioned on the phylogenetic tree, could be a sign of adaptation to specific ecological niches or environmental conditions (Fig. 1, 2). This signifies that this species has undergone more genetic changes over time as indicated by mutation rates, potentially due to various factors such as environmental cues, adaptation, and genetic diversity [67]. In a study, Futahashi et al. [68] disclosed that the genomes of certain dragonfly species contain as many as 33 distinct opsin genes. Although this does not necessarily imply that they possess the ability to perceive 33 distinct colours, the presence of excess copies of certain genes could facilitate evolutionary advancements and modifications to these genes without jeopardising their colour vision due to mutations [68]. Further analysis would be needed to understand the specific implications of this mutation pattern in the context of the study for *T. tillarga*.

Finally, the current investigation establishes a comprehensive DNA barcode database for the accurate identification of the thirteen species of dragonflies from Bangladesh. Genetically distinct from the other species examined, *T. tillarga* may also serve as a useful model organism for future research into a variety of biological phenomena, from color vision to controlling pest insects.

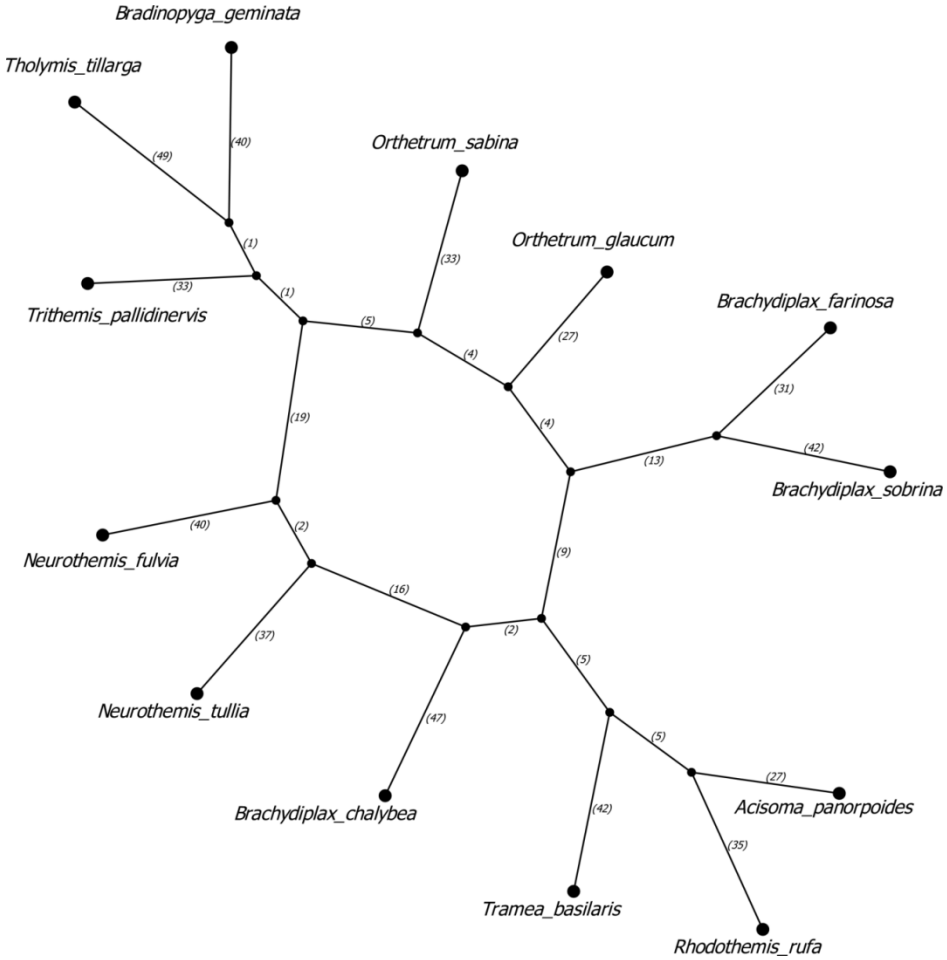

***Fig. 2**. Mitochondrial COI gene-based TCS haplotype network of thirteen dragonflies. The haplotype is symbolized by large circles, while the immediate common ancestors are represented by small circles. The numbers present mutational steps.*

CONCLUSION

The current investigation involved the synthesis of COI gene sequences from thirteen species belonging to the Libellulidae family. Based on BLAST analysis, twelve dragonfly species' COI gene sequences were 98% to 99% identical. The only species without a GenBank match was *B. farinosa*. All thirteen sequences were submitted to GenBank database, where *B. farinosa*'s submission was the first. Then characterization of the COI gene is performed where there was a significant AT bias (65.26%) among dragonfly species recorded. Genetic diversity and evolutionary links among dragonfly species were examined using pairwise distance analysis and phylogenetic tree. The thirteen dragonflies diverged 0.119-0.231% genetically. A Neighbor-Joining (NJ) phylogenetic tree showed two major clades and that all dragonfly species shared a common ancestor. These two clades include all twelve species except *T. tillarga*. The genetic relatedness of these thirteen dragonflies was revealed via a TCS haplotype network, with *T. tillarga* having the most mutations. This result suggests that *T. tillarga* has experienced significant genetic adaptation over time and may serve as a useful model organism for future research into numerous biological events. Furthermore, forthcoming investigations in the field of molecular taxonomy may utilize current findings to identify dragonfly species and unveil a more profound evolutionary connection within the Libellulidae family.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: M.M.H., Design: M.M.H., Data Collection or Processing: A.G., F.S.B., M.K.H., M.A.A.M., M.S.A., Analysis or Interpretation: M.S.A., M.A.A.M., M.M.H., Literature Search: S.M.M., K.A., S.I., Writing: M.M.H., M.S.A.

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REFERENCES

[1] Bashar, K., Reza, M.S., Razzak, M.A., Rahman, K.M.Z., Goda, P., Howlader, A.J. (2014): Faunistic study of Odonata (Dragonfly & Damselfly) in some selected regions of Bangladesh. Journal of Entomology and Zoology Studies 2 (4): 1-6.

[2] Khan, M.K. (2015a): *Gynacantha subinterrupta* Rambur, 1842: an addition to the odonates (Insecta: Odonata: Aeshnidae) of Bangladesh. Journal of Threatened Taxa 7(10): 7704-7705.

[3] Khan, M.K. (2015b): Dragonflies and damselflies (Insecta: Odonata) of the northeastern region of Bangladesh with five new additions to the Odonata fauna of Bangladesh. Journal of Threatened Taxa 7(11): 7795-7804.

[4] Habib, M.S.A., Hossain, M.M., Iqbal, K.F., Khan, T., Islam, M.S., Saif, S., Jewel, M.M.M. (2016): Dragonflies and Damselflies of Bangladesh, Inventory-First Phase. Jiban Bikash Karjocrom. 96 pp.

[5] Subramanian, K.A., Babu, R. (2017): Checklist of Odonata (Insecta) of India. Zoological Survey of India. Version 3.0. 1-51.

[6] Kalkman, V.J., Babu, R., Bedjanič, M., Conniff, K., Gyeltshen, T., Khan, M.K., Subramanian, K.A., Zia, A., Orr, A.G. (2020): Checklist of the dragonflies and damselflies (Insecta: Odonata) of Bangladesh, Bhutan, India, Nepal, Pakistan and Sri Lanka. Zootaxa 4849 (1): 1-84.

[7] Walia, G.K., Singh, H. (2022): A review on intraspecific karyomorphological variations of m chromosomes in family Libellulidae (Anisoptera: Odonata). The Journal of Basic and Applied Zoology 83: 47.

[8] Mitra, A., Thinley, P. (2005): The Odonata of Bumdeling National Park. Department of Forests and Park Services, Ministry of Agriculture and Forests.

[9] Kenny, L.P., Burne, M.R. (2001): A Field Guide to the Animals of Vernal Pools. Natural Heritage and Endangered Species Program. Westborough, MA.

[10] McGeoch, M.A. (1998): The selection, testing and application of terrestrial insects as bioindicators. Biological Reviews 73:181-201.

[11] Simaika, J.P., Samways, M.J. (2012): Using dragonflies to monitor and prioritize lotic systems: a South African perspective. Org Divers Evol 12: 251-259.

[12] Li, L., Zheng, B., Liu, L. (2010): Biomonitoring and Bioindicators Used for River Ecosystems: Definitions, Approaches and Trends. *Procedia Environmental Sciences 2*: 1510-1524.

[13] Buss, D.F., Carlisle, D.M., Chon, T.S., Joseph, C., Harding, J.S., Hanneke, E.K., Wayne, A.R., Stephanie, S., Christa, T., Robert, M.H. (2015): Stream biomonitoring using macroinvertebrates around the globe: a comparison of large-scale programs. Environ Monit. Assess 187(1): 4132.

[14] Brunelle, P.M. (1999): Distribution of Damselflies and Dragonflies (Odonata) of Maine, United States. *Northeastern Naturalist* 6 (2): 95-118.

[15] Hebert, P.D.N., Cywinska, A., Ball, S. L., deWaard, J.R. (2003a): Biological identifications through DNA barcodes. Proceedings of the Royal Society B. 270 (1512): 313–321.

[16] Bady, P., Doledec, S., Fesl, C., Gayraud, S., Bacchi, M., Scholl, F. (2005): Use of invertebrate traits for the biomonitoring of European large rivers: the effects of sampling effort on genus richness and functional diversity. Freshwater Biology 50, 159-173.

[17] Zhou, X., Robinson, J.L., Geraci, C.J., Parker, C.R., Flint Jr, O.S., Etnier, D.A., Hebert, P.D.N. (2011): Accelerated construction of a regional DNA-barcode reference library: Caddisflies (Trichoptera) in the Great Smoky Mountains National Park. Journal of the North American Benthological Society 30(1): 131-162.

[18] Joly, S., Davies, T.J., Archambault, A., Bruneau, A., Derry, A., Kembel, S.W., Peres-Neto, P., Vamosi, J., Wheeler, T.A. (2014): Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. Molecular Ecology Resources 14(2): 221-232.

[19] Koroiva, R., Gomes, V.G.N., Vilela, D.S. (2022): DNA Barcoding and New Records of Odonates (Insecta: Odonata) from Paraíba State, Brazil. Diversity 14: 203.

[20] Lam, Y.T., Kamarudin, K.R, Zakaria, M.Z., Omar, M.S.S., Tokiman, L., Jahari P.N.S., Salleh, F.M. (2021): Mitochondrial Barcodes of Dragonflies and Damselflies Originated from Taman Negara Endau Rompin, Johor, Malaysia. International Conference on Biodiversity: Earth and Environmental Science 736: 012032.

[21] Almansoori, N., Enan, M.R., Al-Deeb, M.A. (2019): Barcode of Life Data Systems (BOLD) Versus GenBank Molecular Identification of a Dragonfly from the UAE in Comparison to the Morphological Identification. OnLine Journal of Biological Sciences 19 (4): 237-244.

[22] Ahmed, H.K., Kareem, D.K. (2024): Molecular identification of some species of dragonfly (Odonata: Anisoptera) using COXI gene and DNA barcodes from Basrah Province, Southern Iraq. Journal of Aquatic Research and Sustainability 1 (1): 8-14.

[23] Waage, J.K. (1979): Dual function of the damselfly penis: sperm removal and transfer. Science 203: 916-918.

[24] Hadrys, H., Schierwater, B., Dellaporta, S.L., Desalle, R., Buss, L.W. (1993): Determination of paternity in dragonflies by Random Amplified Polymorphic DNA fingerprinting. Molecular Ecology 2: 79-87.

[25] Hadrys, H., Schroth, W., Schierwater, B., Streit, B., Fincke, O.M. (2005): Tree hole odonates as environmental monitors: Non-invasive isolation of polymorphic microsatellites from the neotropical damselfly *Megaloprepus caerulatus*. Conservation Genetics 6: 481-483.

[26] Corbet, P.S. (2004): Dragonflies: Behaviour and Ecology of Odonata. Harley Books 829 pp.

[27] Turgeon, J., Stoks, R., Thum, R.A., Brown, J.M., Mcpeek, M.A. (2005): Simultaneous Quaternary radiations of three damselfly clades across the Holarctic. The American Naturalist 165: E78-E107.

[28] Córdoba-Aguilar, A. (2008): Dragonflies and damselflies: Model organisms for ecological and evolutionary research. Oxford: Oxford University Press.

[29] Karthika, P., Vadivalagan, C., Gunasekaran, C., Anandhakumar, S. (2012): DNA Barcoding of selected dragonfly species (Libellulidae and Aeshnidae) for species authentication with phylogenetic assessment. European Journal of Experimental Biology 2 (6): 2158-2165.

[30] Islam, S.U., Qasim, M., Lin, W., Islam, W., Arif, M., Ali, H., Du, Z., Wu, Z. (2018): Genetic interaction and diversity of the families Libellulidae and Gomphidae through COI gene from China and Pakistan. Acta Tropica 182: 92-99.

[31] Chiriboga-Ortega, R., Heyden, C.V., Burgos, M.E.S., Ortega, S., Oña, T., Velarde, E., Goethals, P., Alfaro-Núñez, A. (2021): Molecular diversity of dragonflies in three high- elevation Andean tropical lakes through DNA barcoding. Biotropica 00:1–5.

[32] Carle, F.L., Kjer, K.M., May, M.L. (2015): A molecular phylogeny and classification of Anisoptera (Odonata). Arthropod Systematics & Phylogeny 73 (2): 281-301.

[33] Nisar, M.M., Muhammad, K., Mehmood, S.A., Ahmed, S., Murtaza, B.N., Nadeem, M.S. (2021): Morphological and phylogenetic evaluation of Libellulidae dragonflies from district Attock, Punjab, Pakistan. Intl J Agric Biol 26: 393-400.

[34] Yong, H., Lim, P.E., Tan, J. (2014): Molecular phylogeny of Orthetrum dragonflies reveals cryptic species of *Orthetrum pruinosum*. Scientific Reports 4: 5553.

[35] Fraser, F.C. (1933): Fauna of British India, including Ceylon and Burmah. Odonata. Vol. I. London: Taylor & Francis 433 pp.

[36] Fraser, F.C. (1934): Fauna of British India, including Ceylon and Burmah. Odonata. Vol. II. London: Taylor & Francis 398 pp.

[37] Fraser, F.C. (1936): Fauna of British India, including Ceylon and Burmah. Odonata. Vol. III. London: Taylor & Francis 461 pp.
[38] Mitra, T.R. (2006): Handbook on Common Indian Dragonflies (Insecta: Odonata). Published by Zoological Survey of India ZSI. 124 pp.
[39] Lahiri, A.R. (1987): Studies on the Odonate Fauna of Meghalaya. Zoological Survey of India, 402 pp.
[40] Brower, A.V.Z. (1996): A new mimetic species of *Heliconius* (Lepidoptera: Nymphalidae), from southeastern Colombia, as revealed by cladistic analysis of mitochondrial DNA sequences. Zoological Journal of the Linnean Society 116: 317-332.
[41] Hall, T.A. (1999): Bioedit- A user-friendly biological sequences alignment editor and analysis program for windows95/98/NT. Nucleic Acids Symposium Ser. 41: 95-98.
[42] Kimura, M. (1980): A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111-120.
[43] Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018): MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35: 1547-1549.
[44] Saitou, N., Nei, M. (1987): The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.
[45] Win, N.Z., Choi, E.Y., Jang, D.J., Park, J., Park, J.K. (2015): Molecular comparison of the genus *Junonia* (Lepidoptera: Nymphalidae) in Myanmar. Journal of Asia-Pacific Biodiversity 8(4): 287-294.
[46] Matt, H., Velasco, J. (2022): Phylogenetic Inference", *The Stanford Encyclopedia of Philosophy* (Fall 2022 Edition), Edward N. Zalta & Uri Nodelman (eds.).
[47] Kapli, P., Flouri, T., Telford, M.J. (2021): Systematic errors in phylogenetic trees. Current Biology 31(2): R51-R66.
[48] Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P. (1994): Evolution, Weighting and Phylogenetic Utility of Mitochondrial Gene Sequences and a Aompilation of Conserved Polymerase Chain Reaction Primers. Ann. Entomol. Soc. Am. 87: 651-701.
[49] Clary, D.O., Wolstenholme, D.R. (1985): The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organization, and genetic code. Journal of Molecular Evolution 22(3): 252-271.
[50] Crozier, R.H., Crozier, Y.C. (1993): The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. Genetics 133(1): 97-117.
[51] Avise, J.C. (2000): Phylogeography. The history and formation of species. Cambridge, MA. 447 pp.
[52] Hebert, P.D.N., Ratnasingham, S., deWaard, J.R. (2003b): Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. B. 270 (Suppl): 96-99.
[53] Zhao, X.C., Dong, J.F., Tang, Q.B., Yan, Y.H., Gelibic, I., Van Loon, J.J.A., Wang, C.Z. (2005): Hybridization between *Helicoverpa armigera* and *Helicoverpa assulta* (Lepidoptera: Noctuidae): Development and morphological characterization of F1 hybrids. Bull. Entomol. Res 95: 409-416.

[54] Wu, H., Cao, L., He, M., Han, R., De Clercq, P. (2021): Interspecific Hybridization and Complete Mitochondrial Genome Analysis of Two Ghost Moth Species. Insects 12: 1046.
[55] Sánchez-Guillén, R.A., Vega-Sánchez, Y.M., Sánchez-Herrera, M. (2022): Genetic structure, cryptic species, and hybridization: causes and evolutionary consequences in Odonata', in Alex Cordoba-Aguilar, Christopher Beatty, and Jason Bried (eds), *Dragonflies and Damselflies: Model Organisms for Ecological and Evolutionary Research*, 2nd edn (Oxford, 2022; online edn, Oxford Academic, 15 Dec. 2022).
[56] Phillips, P., Swanson, B.J. (2018): A genetic analysis of dragonfly population structure. Ecol Evol 8(14): 7206–7215.
[57] Cancellario, T., Miranda, R., Baquero, E., Fontaneto, D., Martínez, A., Mammola, S. (2022): Climate change will redefine taxonomic, functional, and phylogenetic diversity of Odonata in space and time. npj Biodiversity 1 (1): 1-14.
[58] Hennig, W. (1981): Insect Phylogeny. Chichester, John Wiley 514 pp.
[59] Carle, F.L. (1982): Evolution of the odonate copulatory process. Odonatologica 11: 271-286.
[60] Carle, F.L. (1995): Evolution, taxonomy and biogeography of ancient *Gondwanian libelluloids*, with comments on anisopteroid evolution and phylogenetic systematics. Odonatologica 24: 383-424.
[61] Nel, A., Martínez Delclòs, X., Placher, J.C., Henrotay, M. (1993): Les 'Anisozygoptera' fossiles – Phylogénie et classification (Odonata). Martinia Numéro Hors-Série 3: 1-311.
[62] Carle, F.L., Kjer, K.M. (2002): Phylogeny of Libellula Linnaeus (Odonata: Insecta). Zootaxa 87: 1-18.
[63] Rehn, A.C. (2003): Phylogenetic analysis of higher-level relationships of Odonata. Systematic Entomology 28: 181-239.
[64] Blanke, A., Greve, C., Mokso, R., Beckman, F., Misof, B. (2013): An updated phylogeny of Anisoptera including formal convergence analysis of morphological characters. Systematic Entomology 38: 474 – 490.
[65] Nisar, M.M., Muhammad, K., Mehmood, S.A., Ahmed, S., Murtaza, B.N., Nadeem, M.S. (2021): Morphological and Phylogenetic Evaluation of Libellulidae Dragonflies from District Attock, Punjab, Pakistan. Intl J Agric Biol 26(3): 393-400.
[66] Laltanpuii, K., Lalremsanga, H.T., Babu, R., Senthil, K.N., Thomas, M. (2017): Distribution and Diversity of Libellulidae (Odonata:Anisoptera) from Indo-Burma Biodiversity Hotspot Region and their Phylogenetic Organization. Research & Reviews: Journal of Zoological Sciences 5 (1): 44-51.
[67] Leigh, W., Bryant, D. (2015): POPART: full-feature software for haplotype network construction Jessica. Methods in Ecology and Evolution 6: 1110–1116.
[68] Futahashia, R., Kawahara-Mikib, R., Kinoshitac, M., Yoshitaked, K., Yajimab, S., Arikawa, K., Fukatsu, T. (2015): Extraordinary diversity of visual opsin genesin dragonflies. PNAS E1247–E1256.